PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF MONOCYTIC MICROPARTICLES (mMP) DERIVED FROM U937 AND THP-1 CELL LINES

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

~	Approximately
%	Percentage
ºC	Degree Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
APC	Antigen presenting cells
aPTT	Activated partial thromboplastin time
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CD	Cluster of differentiation
CMP	Common myeloid progenitors
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
CO_2	Carbon dioxide
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

g	Gram
GMP	Granulocyte-macrophage progenitors
H_2SO_4	Hydrosulfuric acid
HSC	Haematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
Ig	Immunoglobulin
IL-1β	Interleukin-1 beta
L	Litre
LMP	Leukocyte microparticles
LPS	Lipopolysaccharides
mAb	Monoclonal antibodies
MDP	Macrophage and dendritic cell precursors
MFI	Mean fluorescent intensity
mg	Milligram
mL	Millilitre
mm	Millimetre
mMP	Monocytic microparticles
MP	Microparticles
ng	Nanogram
nm	Nanomolar
NMP	Neutrophil microparticles

NTA	Nanoparticle tracking analysis
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PS	Phosphatidylserine
PT	Prothrombin time
PSG	Penicillin, streptomycin with glutamine
PSGL-1	P-selectin glycoprotein ligand-1
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI-1640	Rosewell-Park Memorial Institute-1640
SEM	Standard error of the mean
SSC	Side scatter
TF	Tissue factor
$TF^{+}MP$	Tissue factor-bearing microparticles
TFPI	Tissue factor pathway inhibitor
THP-1 mMP	THP-1 monocytic microparticles
TLR	Toll-like receptor
TM	Thrombomodulins

TNF-α	Tumor necrosis factor alpha
U937 mMP	U937 monocytic microparticles
VCAM	Vascular cell adhesions molecules
VGEF	Vascular endothelial growth factors

PENCIRIAN FENOTIP DAN FUNGSI MONOSITIK MIKROPARTIKEL (mMP) YANG DIREMBES OLEH SEL TERBITAN U937 DAN THP-1

ABSTRAK

Monositik mikropartikel (mMP) ialah partikel membran berdiameter 0.1-1.0 µm yang berasal daripada monosit manusia. Ianya dirembes semasa keadaan fisiologi badan yang normal seperti pengaktifan sel-sel dan apoptosis atau dalam keadaan berpenyakit. Monositik MP berperanan penting dalam keradangan, fungsi sel endotelium dan pembekuan darah, namun mekanisme mMP dalam situasi tersebut belum diperincikan. Monositik MP boleh dikenalpasti melalui ekspresi-ekspresi antigen sel induknya iaitu CD14 dan fosfatidilserin pada permukaan mMP menggunakan sitometri aliran. Dalam kajian ini, kami menggunakan sel-sel terbitan monositik iaitu U937 dan THP-1 untuk mengkaji rembesan mMP dengan kaedah '*in vitro*'. Sebanyak $1x10^6$ sel/mL dirangsang oleh 1 µg/mL 'lipopolysaccharide' (LPS) atau 10 ng/mL 'recombinant human tumor necrosis factor-α' (TNF-α) selama 18 jam dalam 5% CO_2 inkubator pada suhu 37°C. Kemudian, mMP diasingkan melalui tiga fasa pengemparan berbeza: (i) 500 x*g* untuk mengeluarkan pelet sel, (ii) 1,500 x*g* untuk membersihkan serpihan sel dan (iii) 20,000 x*g* ultra-pengemparan untuk mengumpul mendapan mMP. Objektif kajian ini menumpukan tinjauan awal tentang ekspresi antigen pada permukaan mMP, rembesan sitokin-sitokin yang bersifat pro-keradangan dan potensi mMP dalam pembekuan darah. Berdasarkan teori, populasi mMP adalah populasi yang mengekspreskan Annexin-V bersama CD14

iaitu penanda sel induknya. Hasil kajian kami mendapati mMP terbitan U937 dan THP-1 tidak semestinya mempamerkan ekspresi yang sama seperti sel induknya. Seterusnya, kami mengenalpasti keupayaan mMP sendiri dalam merembes sitokin-sitokin pro-keradangan iaitu 'interleukin-1β' (IL-1β) dan TNF-α serta menguji mMP tersebut sama ada mampu meningkatkan rembesan sitokin-sitokin tersebut daripada sel induknya, U937 dan THP-1. Hasil kajian menunjukkan mMP mampu merembes IL-1β tetapi gagal merangsang perembesan IL-1β yang terhasil daripada pengkulturan bersama sel U937 dan THP-1. Manakala, TNF-α pula didapati mampu dirembes daripada mMP yang terhasil daripada rangsangan TNF-α pada sel U937. Monositik MP tersebut juga mampu merangsang perembesan sel induknya iaitu U937, secara pengkulturan bersama iaitu sebanyak hampir 400 pg/mL sitokin TNF-α. Selain itu, mMP berpotensi sebagai pro-koagulasi sebagaimana dipamerkan oleh penanda CD142 pada kedua-dua mMP serta, ujikaji masa 'prothrombin' menunjukkan semakin banyak mMP, semakin singkat masa yang diperlukan untuk koagulasi berlaku. Sebagai kesimpulan, mMP terbitan daripada pelbagai jenis sel-sel monosit mempunyai ciri-ciri fenotip yang berbeza. Oleh itu, penyelidikan terperinci pada masa hadapan perlu dijalankan untuk mengukuhkan lagi pencirian mMP yang terhasil daripada darah manusia serta kaitan mMP dengan implikasi klinikal terutama dalam bidang penyakit berkaitan keradangan.

PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF MONOCYTIC MICROPARTICLES (mMP) DERIVED FROM U937 AND THP-1 CELL LINES

ABSTRACT

Monocytic microparticles (mMP) are 0.1-1.0 µm membrane particle, shed from human monocytes under physiological condition such as cellular activation and apoptosis or during certain pathological conditions. It has been reported in the literatures that mMP play important roles in inflammation, endothelial cell function and blood coagulation, however, the mechanism involved is still unclear. Monocytic MP can be characterised by the expression of their parental cell antigen, CD14 along with phosphatidylserine (PS) on mMP surface which can be detected by flow cytometry. This study was conducted using U937 and THP-1 monocytic cell lines. To induce mMP production *in vitro,* 1 x 10^6 cells were stimulated with 1 µg/mL lipopolysaccharide (LPS) or 10 ng/mL recombinant human tumor necrosis factor-α (TNF-α) for 18 hours in 5% CO_2 humidified incubator at 37°C. Then, mMP were isolated through three differential centrifugations: (i) 500 x*g* to remove cell pellet, (ii) 1,500 x*g* to remove cell debris and (iii) 20,000 x*g* ultra-centrifugation to pellet mMP. This study was aimed to identify mMP surface marker expression by flow cytometry, measure mMP-induced proinflammatory cytokine secretions and assess coagulation potential of mMP. Theoretically, mMP populations are defined as positive for Annexin-V in combination with monocytic marker, CD14. Our finding has shown that U937 mMP and THP-1 mMP does not necessarily follow the same surface antigen

expression as their parent cells. We then investigated the ability of mMP to secrete proinflammatory cytokines; TNF-α and interleukin 1 beta (IL-1β) and also tested mMP potential to modulate cytokine secretions in U937 and THP-1 cells. We found that IL-1β was secreted from both mMP but was down regulated when co-cultured with U937 and THP-1 cells. TNF-α was secreted from all TNF-α-stimulated samples and only mMP-derived U937 upregulated TNF-α secretion almost up to 400 pg/mL from TNF-α-stimulated U937 cells. Besides that, mMP may have a procoagulant potential as shown by high expression of CD142 on both mMP surface and shorter prothrombin time (PT) over an increasing number of mMP. In conclusion, different monocytic cells may give rise to different properties of mMP. Further study should be carried out to establish a detail characterisation of mMP isolated from human blood and discover the clinical implications of mMP especially in inflammation-related diseases.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Monocytes are a type of white blood cell that participate in the immune responses. They originate from the bone marrow and circulate in the blood within 1–2 days. Upon host exposure to foreign pathogens (e.g., virus, bacteria, fungi), the first line defence system known as innate immune response will be activated to prevent these pathogens from entering the host. However, if the innate immune system is incapable of removing the pathogens, infection might occur and may lead to inflammation (Peter *et al.*, 2012). During infection, monocytes migrate to the infected tissue and differentiate into macrophages, followed by phagocytosis of pathogens. Immune cells are also recruited along with chemoattractants followed by secretion of inflammatory cytokines (Abbas *et al.*, 2011; Lydyard *et al.*, 2011; Peter *et al.*, 2012). Concurrently, microbial products released around the inflamed sites may trigger monocytes to release small membrane vesicles known as monocytic microparticles (mMP) (Baron *et al.*, 2012).

Monocytic microparticles are microvesicles ranging from 0.1 to 1.0 μm in diameter and are secreted from the plasma membrane of monocytes in response to cellular activation and apoptosis (Wu *et al.*, 2013). Due to high phosphatidylserine (PS) expression on microparticles (MP) surface membrane arise from the loss of cell plasma membrane symmetry during MP formation, MP are often characterised as being Annexin V positive, which binds to PS (Burnier *et al.*, 2009). In addition, MP also express cell surface antigens

which highlight their origin, for example, CD14 for monocytes, CD144 for endothelial cells and CD162 for activated T-lymphocytes. Similar to other MP, theoretically mMP can be characterised by the expression of their original cell surface marker, CD14 and PS on their surface membranes (van Ierssel *et al.*, 2010). However, mMP derived from unstimulated monocytes was reported to possess undetectable CD14 expression (Crompot *et al.*, 2015).

Currently, research on mMP has gained interest as they have been shown to participate in coagulation (Wen *et al.*, 2014), inflammation (He *et al.*, 2017) and cell-to–cell communication as well as being an indicator for certain pathological conditions (Walsh *et al.*, 2017). Monocytic MP are abundant in many pathological settings and their number is reported to increase in infection (Mack *et al.*, 2000; Ling *et al.*, 2011), endothelial cells function (Wang *et al.*, 2011) and immune-mediated diseases (Distler *et al.*, 2005a; Takeshita *et al.*, 2014). In a healthy individual, circulating mMP exist more likely due to apoptosis process from aging (Zubairova *et al.*, 2015). To date, the role of mMP in the circulation during certain pathological conditions may either assist in progressing or limiting the diseases, or simply function as biomarkers (Halim *et al.*, 2016). Even though many studies of MP have been reported, the exact function and importance of mMP in physiological conditions is not clear. Hence, a consistent characterisation and detection criteria of mMP is required.

1.2 Rationale of the study

Research interest in MP has increased over the past decades. To date, many researches have focused on MP derived from endothelial cells and platelets as well as their role in vascular biology (Hoyer *et al.*, 2012). In this study, U937 and THP-1 monocytic cell lines were used as monocyte models due to their ability to mimic monocytes. These monocytic cell lines are easier to be cultured in a large quantity compared to primary monocytes isolated from human blood. It has been reported previously that mMP derived from LPS-stimulated THP-1 play a role in inflammation (Wen *et al.*, 2014), thrombin and fibrin generation, coagulation (Aleman *et al.*, 2011) and endothelial cell function (Wang *et al.*, 2011). To date, information on mMP characterisation derived from U937 and THP-1 is still lacking while the role of mMP derived from primary monocytes is also unclear. Monocytic MP have been shown to secrete pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 (Distler *et al.*, 2005b; Cerri *et al.*, 2006; Wen *et al.*, 2014). A previous study has shown that interleukin-1β, a proinflammatory cytokine secreted from LPS-stimulated mMP, was able to aid endothelial cell activation and thus amplify inflammation (Wang *et al.*, 2011). However, they did not assess the ability of mMP in upregulating monocytes to increase the secretion of proinflammatory cytokine in an autocrine manner as suggested by other literatures (Bardelli *et al.*, 2012; Schildberger *et al.*, 2013).

Our study focused on characterising mMP phenotypes derived from LPS- and TNF-α-stimulated U937 and THP-1 monocytic cells through differential centrifugation steps. To assess mMP function, we tested the coagulation potential of mMP and cytokine secretion through prothrombin time assay and enzyme-linked immunosorbent assay (ELISA)

respectively. We hypothesised that mMP derived from LPS- and TNF-α-stimulated U937 and THP-1 cells may differentially modulate cell surface expression, proinflammatory cytokine secretion, and coagulation properties, given that different types of monocytic cell lines have unique phenotype and function.

1.3 Objectives of the study

1.3.1 General objective

To characterise surface phenotypes and functions of monocytic microparticles derived from U937 and THP-1 monocytic cells following lipopolysaccharide (LPS) and TNF-α stimulations.

1.3.2 Specific objectives

1) To determine cell surface antigen of monocytic microparticles derived from stimulated U937 and THP-1.
2) To measure the level of proinflammatory cytokines (interleukin-1β [IL-1β] and TNF-α) in mMP culture and mMP co-culture with U937 and THP-1.
3) To assess coagulation property of mMP derived from U937 and THP-1.

CHAPTER 2

LITERATURE REVIEW

2.1 Monocytes

2.1.1 Origin, function and classification

Monocytes are part of the immune system that comprise 5% to 10% of total leukocytes. They originate from haematopoietic stem cells (HSC) in the bone marrow before undergoing specific differential stages, for example, common macrophage and dendritic cell precursors (MDP), common myeloid progenitors (CMP), or granulocyte-macrophage progenitors (GMP) (Figure 2.1). After being produced in the bone marrow, inactive monocytes circulate in the blood for 1–2 days before differentiating into either a mature form or undergoing apoptosis. Monocytes can be characterised by their agranular feature with size 15 to 20 μm in diameter, kidney-shaped nucleus, and cytoplasmic vesicles (Akashi *et al.*, 2000; Fogg *et al.*, 2006; Hettinger *et al.*, 2013).

Monocytes play an important role as an innate effector by initiating inflammatory response towards microbes by phagocytosing pathogens, producing reactive oxygen species (ROS), and secreting inflammatory cytokines. In pathological conditions, monocytes contribute to atherogenesis, angiogenesis, and myeloperoxidase production (Serbina *et al.*, 2008; Avraham-Davidi *et al.*, 2013). In the tissues, monocytes are commonly known as macrophages. The ability of macrophage to phagocytose foreign invaders are important during infection. Macrophages are attracted to infection sites

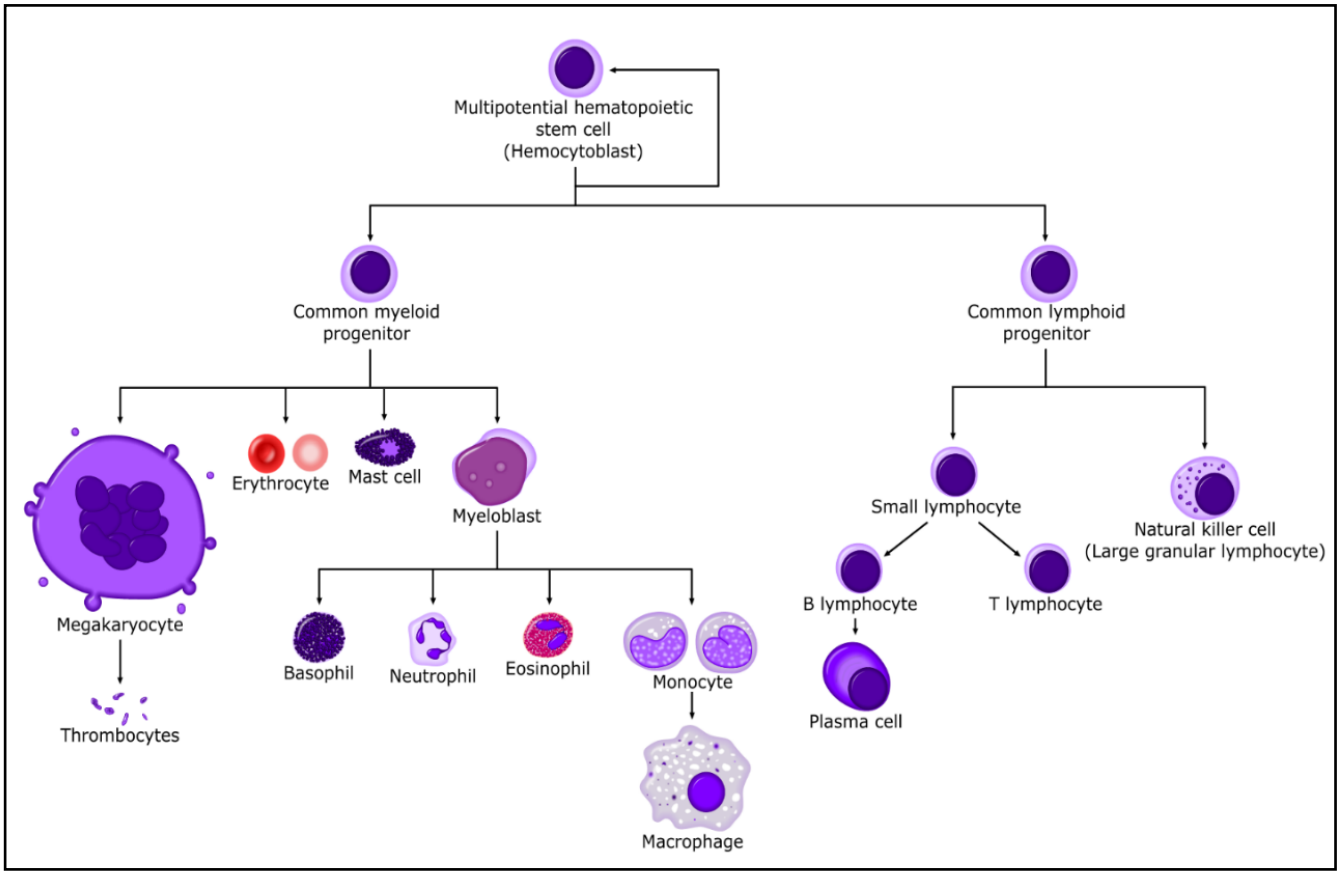

Figure 2.1 Origin of human blood cells. Monocytes are a group of myoblasts which originated from myeloid progenitor. Monocytes, basophils, neutrophils, and eosinophils are known as leukocytes, which play a major role in the human defence system. Activated monocytes differentiate into macrophages which function as antigen-presenting cells (APC). Adapted from Shaikh and Bhartiya (2012).

via chemotaxis, adhere to pathogens, ingest them and subsequently destroy the pathogens (Lydyard *et al.*, 2011). This mechanism needs an interaction between the surface receptor (pattern recognition receptors, PRR) of monocytes/macrophages and the pathogen-associated molecular patterns (PAMP) of microbes. Examples of PAMP that can be recognised by monocytes are bacterial lipopolysaccharides (endotoxin), terminal mannose residues on bacterial glycoproteins, and CpG (cytosine-guanine) nucleotide in bacterial DNA. The PRR such as the toll-like receptor, Fc receptor, and adhesion receptor, allow monocytes/macrophages to detect and recognise various pathogens (e.g., bacteria, parasites, and worms) and subsequently releasing inflammatory mediators to trigger the adaptive immune responses (Peter *et al.*, 2012).

Human blood monocytes are classified according to their surface expression of CD16 and CD14 antigens. Monocyte subsets can be subdivided into classical monocytes ($CD14^{++}$ $CD16^{-}$) that comprise 90% of total monocytes, 10% intermediate monocytes ($CD14^{++}$ $CD16^{+}$) and relatively low amount of non-classical monocytes ($CD14^{dim}$ $CD16^{++}$), as reported by the Nomenclature Committee of the International Union of Immunological Societies (Italiani and Boraschi, 2014; Angelovich *et al.*, 2015). Nevertheless, the physiological role of these monocyte subsets *in vivo* was not fully elucidated. Different monocyte subsets have different roles during homeostasis, immune defense, inflammation, and tissue repair, in terms of their capacity to become activated and secrete inflammatory cytokines in response to different stimuli, antigen processing and presentation (Italiani and Boraschi, 2014).

2.1.2 Monocytic cell lines

Cell lines are preferred by most researchers because of their ability to grow to a large number within a short period of time compared to primary cells. Monocytic cell lines are a group of cell population that phenotypically and genetically resemble human primary monocytes (Daigneault *et al.*, 2010). Monocytic cell lines are easily accessible compared to blood monocytes without the requirement of isolation steps from blood. Furthermore, inaccurate prediction of the actual role of monocytes arising from interference with other blood components can be avoided due to the homogeneity of the cell lines (Shet, 2008).

There are numerous monocytic cells namely U937, THP-1, HL-60, ML-2, and Mononomac-1. They have an extended life span by continuous passage within a long time (Chanput *et al.*, 2015) and are commonly studied for cell proliferation and differentiation into macrophages by using different stimuli such as phorbol-12-myristate-13-acetate (PMA), 1,25-dihydroxyvitamin D3 (VD3), ionomycin, and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Yazdanparast *et al.*, 2006; Daigneault *et al.*, 2010; Posada *et al.*, 2015). In addition, the use of cell lines enables the study of macrophage function because primary macrophages are difficult to isolate and grown *ex vivo*. Although *in vitro* differentiation of macrophage may not resemble the actual *in vivo* condition, the use of cell lines are still preferred due to the availability and easy to grow (Daigneault *et al.*, 2010).

2.1.2 (a) THP-1 and U937

THP-1 and U937 are single-rounded monocytic cells that exist in a suspension during early culture state. THP-1 is a leukaemic cell line derived from of an acute monocytic leukaemic patient in 1980 (Tsuchiya *et al.*, 1980). THP-1 cells are widely used to study monocyte functions as they represent mature monocyte phenotypes and carry homogenous genetic behaviours (Qin, 2012). A number of studies have been carried out using THP-1 as a model such as in genetic study (Gillies *et al.*, 2012) In addition, LPS-stimulated THP-1 cells have been reported to activate and improve endothelial cell interactions similar to primary monocytes (Schildberger *et al.*, 2013). The THP-1 gene expressions are also similar to peripheral blood mononuclear cells (PBMC)-derived macrophages (Sharif *et al.*, 2007).

U937 is a pro-monocyte cell line isolated from a 37-year-old Caucasian man with histiocytic lymphoma (Sundström and Nilsson, 1976). U937 also represents a human macrophage-like model (Liu *et al.*, 2012) in several macrophage differentiation assays. U937 has been demonstrated to participate in the mechanism of monocyte-endothelium attachment and has been previously studied for biological effect of particles (Posada *et al.*, 2015) as well as cytokine release (Daigneault *et al.*, 2010).

Generally, U937 cells are stable as they can be used at a higher passage number compared to THP-1 which is stable only until passage 25 (Chanput *et al.*, 2015) . In a long-term cryopreservation, U937 shows a stable recovery without any noticeable effect on monocyte features. THP-1 and U937 cells are commonly studied for their gene

transcription and cytokine production by using polymerase chain reaction (PCR), Western blot, ELISA and flow cytometry (Chanput *et al.*, 2015).

2.2 Microparticles

Microparticles (MP), exosomes, and apoptotic bodies are extracellular vesicles released from cells (Théry *et al.*, 2009). They can be distinguished based on different sizes, mechanism of vesicle formations as well as protein and lipid membrane contents (Table 2.1). The sizes of apoptotic bodies and exosomes are approximately more than 1000 nm and 30–100 nm respectively. Apoptotic bodies are produced by cell death events while exosomes are formed by fusion of small fragment multivesicular bodies which are different from the profile of MP (Burger *et al.*, 2013).

Microparticles were first detected in 1967 and were known as platelet dust (Wolf, 1967). They are small membrane-bound particles of 0.1 to 1.0 μm in diameter (Wu *et al.*, 2013). Under physiological conditions, MP are continuously released from their cells of origin during cellular apoptosis and following cellular activation by various stimuli. They are heterogeneous in size, composition, cellular origin and density (Zubairova *et al.*, 2015). They carry antigens of their origin cells and transport them from the MP surface to other cells and organs (Mause and Weber, 2010).

Table 2.1 Characteristics of extracellular vesicles

	Exosomes	**Microparticles**	**Apoptotic bodies**
Size	10-100 nm	100-1000 nm	>10000nm
Formation mechanism	Fusion of multivesicular bodies with plasma membrane	Outward blebbing of plasma membrane	Late apoptosis
Detection method	Electron microscopy and Western blot	Flow cytometry, capture-based assay and electron microscopy	Flow cytometry, capture-based assay and electron microscopy
Characteristic features	LAMP1, CD63 and TSG101	High PS expression and cell-specific surface markers	High PS expression, DNA and permeable membrane
Composition	Protein, RNA, miRNA	Protein, RNA, miRNA	Protein, DNA, cell organelles, RNA and miRNA
Membrane properties	Rich in lipid raft and impermeable	Externalised phosphatidylserine, rich in lipid raft and impermeable	Externalised phosphatidylserine and permeable

Adapted from Burger *et al*., (2013).

Microparticles exist in circulating blood under normal conditions. However, under pathological conditions, MP levels are linked to disease severity. Microparticle levels are elevated in conditions associated with homeostasis, inflammation, cell survival and apoptosis, vascular remodelling, angiogenesis, endothelial function, infection, and immune-mediated diseases. Many studies have reported that MP contribute to pathological processes, thus MP may act as a potential biomarker and mediator of various diseases (Burnier *et al.*, 2009; Roos *et al.*, 2010; Zhang and Yang, 2012).

The membrane compositions of MP reflect their membranous elements from their original cell. They convey genetic storage (e.g., microRNA, mRNA), cytoplasmic components such as adhesion molecules, membrane phospholipids, and specific antigens (Table 2.2) that arise from the cell of origin. The membrane compositions of MP also rely on the type of *in vitro* stimuli received from mother cells (Nomura and Shimizu, 2015).

Table 2.2 List of antigens that are expressed on microparticles derived from erythrocytes, platelets, neutrophils, monocytes, lymphocytes and endothelial cells.

Origin Cell	**Antigens**	**Stimuli on origin cell**
Erythrocytes	CD35, CD235a (glycophorin A)	A23187, pH, ATP depletion, decompression stress, diamide
Endothelial cells	CD31 (platelet endothelial cell adhesion molecules), CD144	Shear stress, uremic toxins, CRP, Ang II, High Glucose, ROS, thrombin, IL-1α, TNF-α, LPS, PAI-1
Platelets	CD235a (glycophorin A) CD42a (GPIX) CD42b (GPIb) CD41 (GPIIb/IIIa, αIIb β3) CD61 (GPIIIa) CD62P (P-selectin)	Shear stress, exercise, pregnancy, CD40L, ADP, thrombin, collagen, apoptosis, A23187, calcium, TRAP, PMA, Cytochalasin D, LPS, epinephrine
Monocytes	CD14 (endotoxin receptor)	LPS, A23187, etoposide, TNF-α, Fas ligand
Lymphocytes	CD4, CD8, CD20	Hepatitis C, storage, actinomycin D, PHA, etoposide, staurosporine, TNF-α
Neutrophils	CD66b (CEACAM-1) CD51 (vitronectin receptor, αv β3)	PMA, Bacterial infection, ANCA, FMLP

Adapted from Nomura and Shimizu (2015).

2.2.1 Origin and structure of microparticles

Microparticles are derived from almost all eukaryotic cells such as erythrocytes, leukocytes, platelets, endothelial cells, tumour cells, and smooth muscle cells (Pliyev *et al.*, 2014). Circulating MP from blood cells are the most reported in the literatures, in particular, platelet- and endothelial-derived MP (Amin *et al.*, 2007; Burnier *et al.*, 2009; Baron *et al.*, 2012). One of the noticeable features of MP is the exposure of phosphatidylserine (PS) on their surface which can be detected by Annexin-V binding (Figure 2.2).

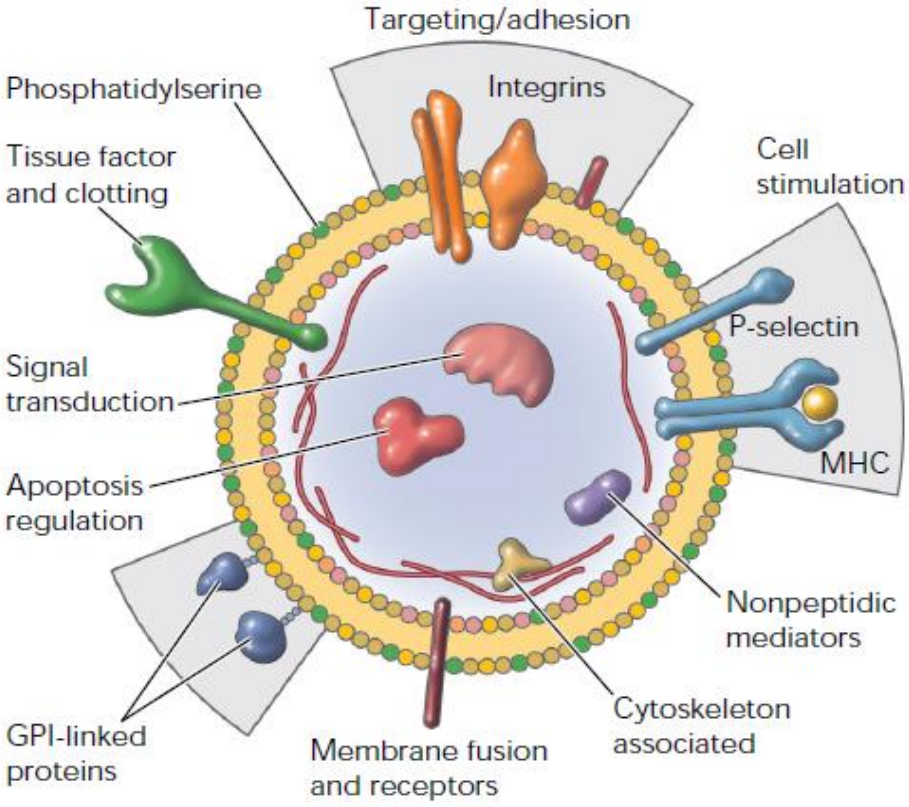

Figure 2.2 Structure and cellular composition of microparticle Various types of membrane proteins and receptors are exposed on a proposed microparticle structure as illustrated by Wu *et al.*, (2013). Cytoplasmic contents within microparticles carry genetic materials from parent cells.

2.2.2 Formation and function of microparticles

Microparticles are shed from their cell membrane by vesiculation and budding through physiological conditions such as cell activation, apoptosis or tissue damage. Their secretion can also be induced *in vitro* using stimulants such as LPS, TNF-α, calcium ionophore and histamine (Mastronardi *et al.*, 2011; Neri *et al.*, 2011; Bardelli *et al.*, 2012). Phosphatidylserine is a negatively-charged lipid membrane residue that arises from loss of membrane integrity due to calcium influx and cytoskeletal changes resulting from cellular apoptosis and activation (Owens and Mackman, 2011). The protrusion of PS on a lipid raft of a cell membrane is caused by the flip-flopping of lipid bilayer controlled by flippase, floppase, and scramblase enzymes. Phosphatidylserine externalisation is the result of cytoskeletal rearrangement and phospholipid membrane alteration events; however, the exact mechanism of PS exposure on plasma membrane is not precisely known. In addition, different experimental settings and cellular stimulations, for example, apoptosis stimulants (staurosporine), cellular stress inducers (ROS, serum deficiency, ultraviolet light), inflammatory cytokines (TNF-α) or transcription blockers (actinomycin D), will give rise to different expression level of PS and other surface markers (VanWijk *et al.*, 2003; Wang *et al.*, 2011; Baron *et al.*, 2012).

The level of MP is increased in many clinical settings such as in angiogenesis, thrombosis, haemostasis, and inflammation (Mack *et al.*, 2000; Boulanger *et al.*, 2001; Kim *et al.*, 2004). Microparticles may act as potential biomarkers in pathological conditions and provide early detection of inflammatory diseases which later aid possible therapeutic interventions (Burger *et al.*, 2013). Furthermore, MP enhance cell-to-cell communication

for example, leukocyte microparticles (LMP) activate endothelial cells and transfer leukocyte antigens to epithelial cells while platelet microparticles (PMP) modulate monocyte-endothelial cell interactions and stimulate proliferation, survival, adhesion and chemotaxis of leukocytes. These demonstrate that MP can be an efficient vehicles of biological information from one cell type to another (Hugel *et al.*, 2005). The function of MP is carried out by MP surface receptor and bioactive molecules including cytoplasmic contents such as cytokine, nucleic acids, and signal proteins. In addition, MP also play an important role in signal transduction by sensing bioactive molecules through surface receptors which later modulate cellular responses and contribute to innate and adaptive immunity (Hugel *et al.*, 2005). Microparticles either stimulate or suppress immune response by secreting pro- or anti-inflammatory cytokines. (Distler *et al.*, 2005b)

2.2.3 Leukocytes-derived microparticles (LMP)

Leukocytes-derived microparticles (LMP) are derived from white blood cells which include monocytes/macrophages, neutrophils, and B- and T-lymphocytes. Like other MP, they also carry surface markers and exhibit similar membrane and cytoplasmic proteins from their parent cells. Leukocyte-derived MP are mainly involved in either thrombosis or haemostasis depending on the presence of tissue factor (TF) and coagulation inhibitors. They also regulate inflammatory response by promoting the recruitment of immune cells and also modify endothelial cell functions (Angelillo-Scherrer, 2012)

Neutrophil-derived microparticles (NMP) represent major LMP population as neutrophils make up 40% to 70% of the total circulating leukocytes in human blood. Neutrophils

originate from the bone marrow and have a short life span for approximately few hours. They play their function according to the target cells that they have to interact with. During inflammation, chemotactic signals trigger neutrophils to the infected area. Neutrophil-derived MP are released through exocytosis by activated neutrophils in the case of a bacterial infection or under *in vitro* stimulation using N-formyl-methionyl-leucyl-phenylalanine (FMLP), anti-neutrophil cytoplasmic antibodies (ANCA), or PMA (Andrews and Berndt, 2008). Similar to other MP, NMP also expose PS on their outer membranes. Furthermore, they function as inflammatory mediators to activate the classical and complement pathways by interacting with endothelial cells and macrophages (Mesri and Altieri, 1999). Neutrophil-derived MP interact with resting platelets and assist in thrombin generation which later leads to thrombosis (Pluskota *et al.*, 2008).

Lymphocytes originate from hematopoietic stem cells in the bone marrow. Lymphoid progenitors migrate and mature in the thymus to become T-lymphocytes or remain in the bone marrow and differentiate into B-lymphocytes before entering peripheral blood. They represent 25% to 40% of leukocyte population. In autoimmune patients, the level of MP derived from B-lymphocytes (BMP) together with other LMP are elevated in the bloodstream (Baka *et al.*, 2010). On the other hand, T-lymphocyte-derived microparticles (TMP) have broad functions such as activating nuclear factor-κB (NF-κB), upregulating cyclooxygenase-2 and nitric oxide expression, carrying Fas ligand and interacting with smooth muscle cells (Tesse *et al.*, 2008; Carpintero *et al.*, 2010).

Monocyte-derived microparticles (mMP) is another part of LMP and they are the main focus in this study. Monocytic MP arise from monocytes which constitute 5% to 10% of blood leukocytes. Monocytic MP have been reported to increase endothelial cell thrombogenicity by displaying many effector molecules such as TF, thrombomodulin (TM), activated protein C (APC), and tissue factor inhibitor (TFPI) on their surface (Aharon *et al.*, 2008). Monocytic MP has also been shown to induce apoptosis by transferring caspase-1 as a cell death signal to smooth muscle cells (Sarkar *et al.*, 2009).

Generally, LMP take part in countless physiological processes including angiogenesis, atherosclerosis, endothelial function, inflammation, and thrombosis. Predicting LMP level might be useful as prognostic tools in cardiovascular events and they may exhibit their functional properties depending on the stimuli used for their *in vitro* generation and the type of cell origin (Angelillo-Scherrer, 2012).

2.2.4 Isolation of microparticles

Microparticle isolation involves several methods which depends on the original source of MP either from blood, tissues, body fluid, or cultured cells. Numerous studies have assessed MP generation *in vitro* compared to *ex vivo* due to the limited number of MP production from *ex vivo* blood (Baron *et al.*, 2012). For MP isolation from blood samples, a proper selection of blood tube containing different anticoagulants play a major role in determining PS^+ MP. For instance the use of blood collection tube with sodium citrate as anticoagulant results in significantly higher level of PS^+ MP compared to blood collected in tubes containing heparin as an anticoagulant (Barteneva *et al.*, 2013). However, due to

limited amount of MP generated *ex vivo*, many studies have used cells lines combined with i*n vitro* culture technique in the presence of inflammatory or apoptotic stimuli to induce MP secretion in large quantities. As suggested by a previous study, all cell types can generate MP, however, each cell lines may display different response to apoptotic or inflammatory stimuli and thus differ in their ability to generate MP (Halim *et al.*, 2016). The most often used cell lines are human umbilical vein endothelial cells (HUVEC) and human lymphoid cells which is Jurkat cells (Baron *et al.*, 2012).

Many studies have used differential centrifugation technique to isolate MP from the whole blood or stimulated cell lines as described in the literatures (Dey-Hazra *et al.*, 2010; Barteneva *et al.*, 2013; Wen *et al.*, 2014; Crompot *et al.*, 2015). Briefly, a cell-free supernatant is first prepared through centrifugation at 1,500 ×*g* before being centrifuged at a high speed (~20,000 ×*g*) to pellet MP (Baron *et al.*, 2012). However, it should be considered that filtration and washing steps of MP pellet after the final ultracentrifugation are not recommended as it may reduce the number of events, particularly during flow cytometry analysis. The washing steps and filtration can lead to fragmentation of larger MP (Barteneva *et al.*, 2013; Nielsen *et al.*, 2014). Furthermore, the buffers involved during centrifugation of MP should be filtered and the samples should be analysed either fresh or stored in the same duration as the storage condition of MP. These steps are essential to ensure reproducible MP and minimise the chances of phenotypic modification of MP (Dey-Hazra *et al.*, 2010).

2.2.5 Detection of microparticles

Various methods to identify MP populations and to characterise their phenotypic and proteomic profiles have been reported in the literatures (Table 2.2). These methods include electron microscopy, mass-spectrometry, flow cytometry, and functional assays such as clotting test, enzyme-linked immunosorbent assay (ELISA) as well as light scattering techniques like nanoparticle tracking analysis, dynamic light scattering, and submicron particle analysis. Although a standard measurement procedure of MP characterisation has not been established yet, flow cytometry and microscopy techniques are the most reliable and preferred methods to quantitatively measure MP (Barteneva *et al.*, 2013).

Table 2.3 List of microparticle detection methods

Assays	Methods	Limitations
Electron microscopy	Observe MP morphology including diameter and cross section	May include artefact detection produced from sample preparation
Functional assays (ELISA, procoagulant activity, thrombin generation)	Identify coagulation and prothrombinase of MP	Only a single parameter of biological function is measured each time
Immunoassays	Capture MP based on presence of surface antigen	Quantification of bulk rather than individual MP
Mass spectrometry	Identify multiple protein content in MP	Unable to estimate MP size and involve many starting materials
Light scattering techniques (dynamic light scattering, nanoparticle tracking analysis [NTA])	Clearly visualise MP by size light microscopy and light scattering involving Brownian motion of single MP	Time consuming Non-universal technology
Atomic force microscopy	Using cantilever to examine MP surface	Non-universal technology

Adapted from Barteneva *et al*., (2013) and Burger *et al*., (2013).

2.2.5 (a) Flow cytometry analysis

Flow cytometry is the most widely used technique to identify MP due to its feasible and broad parameters as well as the ability to rapidly enumerate individual MP in many samples (Poncelet *et al.*, 2015). Acquisition in flow cytometry analysis is based on the light scattering and fluorescent intensity produced when a single particle in a suspension form travels through a fluid stream and then recorded by a specialised software (Adan *et al.*, 2017). Theoretically, it quantifies antigens expressed on cell surface by using antibodies that are covalently attached to fluorescent markers. Monoclonal antibodies (mAb) are typically used as they have high specificity and specific epitope for a particular antigen (Lydyard *et al.*, 2011).

Besides measuring surface markers, size-calibration beads can also be used to determine the heterogeneity of MP size (Aass *et al.*, 2011; Crompot *et al.*, 2015). The beads have different sizes, ranging from 300 to 1000 nm in diameter, and are assessed by the forward scatter parameter on a flow cytometry. Size-calibration fluorescent technique is normally performed to identify MP gate on a dot plot as a preliminary standardisation template. However, the drawback of using polystyrene synthetic beads is that they give a higher signal due to refractive index and may lead to false estimation of MP size (Nomura *et al.*, 2002; Nielsen *et al.*, 2014; Crompot *et al.*, 2015).

Several studies have suggested a few technical strategies for MP detection. Firstly, an absolute minimum threshold of 200 at the side scatter (SSC-A) parameter is preferred for MP population to avoid the exclusion of smallest events and minimise the distribution of

instrumental noises (Aass *et al.*, 2011). Secondly, the detection of particle size is set up below 1.0 µm by using size-fluorescent bead and supported with double positive staining of surface marker and PS marker (if applicable) to separate true events from background noises (Aass *et al.*, 2011). Moreover, filtration of staining buffer with 0.22 µm filter membrane and using a low flow rate may be able to distinguish particles from background debris (Ogata *et al.*, 2006; Neri *et al.*, 2011; Hoyer *et al.*, 2012; Nielsen *et al.*, 2014; Walsh *et al.*, 2017).